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Title: Prion test

The invention is related to diagnostic methods for detecting transmissible spongiform encephalopathies (TSEs) such as BSE, scrapie and related diseases in animals and humans.

Bovine spongiform encephalopathy (BSE or mad cow disease)of cattle and scrapie of sheep are fatal, non-inflammatory neurodegenerative diseases caused by prions and are characterized by a long incubation period. In humans Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia and kuru belong to this category of TSEs.

Although scrapie, the prototype of the family of TSEs, in sheep and goats has been known for over 200 years (Pattison, 1988) and has been diagnosed world-wide (with the exception of New Zealand and Australia), it is only since 1986 that BSE has been described in cattle in the UK. By January 1998, there had been 170,259 confirmed cases of BSE in Great Britain and there may exist a great number of cases of not yet overt ("silent") BSE. BSE probably emerged because scrapie-contaminated sheep offal had been included in cattle feeding-stuff via meat and bone meal and newly infected cattle material was then recycled (Wilesmith et al., 1991). This mechanism is quite plausible since ovine scrapie could be transmitted experimentally to several animal species, including cattle (Hourrigan, 1990; Gibbs, 1990). Alternatively, recycling of offal from a rare case of spontaneous BSE for cattle feedstuff could also have led to the BSE epidemic. Moreover, the number of cattle in the UK with BSE reported annually is declining after the ban on feeding meat and bone meal in 1988.

Brain homogenates from cows with BSE produce after inoculation of mice a characteristic pattern of brain lesions in mice. Also characteristic incubation periods in inbred lines of mice are seen. This is identical to the pattern elicited by brain tissue from individuals who recently have died from new-variant Creutzfeldt-Jakob disease (nvCJD; Bruce, 1997). The conclusion is that the BSE agent is identical to the nvCJD agent. Up to now, this variant has caused the death of 35 young Britons and one Frenchman (Will et al, 1996; info: CJD Statistics per 30 November 1998, Internet).

There is also concern that the BSE strain that seems to be transmissible to humans may have infected sheep, where it could produce a disease hardly distinguishable from scrapie. When its ominous strain-specific properties are maintained across the species barrier, sheep BSE may be a threat to human health, although scrapie by itself seems not to transmit to humans. Indeed, BSE agent has been transmitted experimentally to sheep by the oral route (Foster et al., 1993) and thus could have the potential to infect sheep under field conditions. With the exception of a bioassay in mice, no diagnostic method is available to discrimination between BSE and scrapie in sheep at present.

Thus far, the only known component of the infectious prion is an abnormal, disease-causing isoform of the "normal" prion protein (PrP) called PrP^{Sc} or aberrant prion protein. PrP, or normal prion protein, is ubiquitous in mammalian cells in a benign, cellular conformation (PrP^{C}) and is encoded within a single exon as a protein of about 250 amino acid residues (figure 1). The PrP gene has been cloned and sequenced from a variety of species and there is a high degree of structural and organisational homology between mammalian PrP sequences (Schatzl et al., 1995). PrPs in many mammals have a 22-24 residues long N-terminal signal sequence

as well as a 22-24 residues long C-terminal signal sequence for attachment of a GPI-anchor. This glycosyl-phosphatidylinositol linkage is a fairly common means of anchoring membranes of proteins to eukaryotic cells. Further structural characteristics of the mature protein (of 206-210 amino acid residues) are one disulfide bond and two sites for Asn-linked glycosylation.

PrP^{Sc} originates from the normal cellular isoform (PrP^{C}) by a post-translational process since the amino acid sequence of PrP^{Sc} is identical to that predicted from cDNA or genomic nucleic acid sequences. Glycosylation patterns are also identical between PrP^{C} and PrP^{Sc}. Moreover, Caughey & Raymond (1991) demonstrated that PrP^{Sc} is made from a cell surface precursor that is identical to the normal PrP. PrP^{Sc} differs from the normal, membrane bound cellular prion protein by its relative protease resistance. Treatment with proteinase K (PK) for instance, results in complete proteolysis of PrP^{C} whereas in PrP^{Sc} the N-terminal part is removed before the amino acid at position 90 (human numeration). The protease-resistant core left is designated PrP27-30 after its electrophoretic behavior in SDS-PAGE as a protein molecule with M_r = 27-30 kDa, and this molecular species retains full infectivity.

Further distinguishing features of PrP^{Sc} are its thermal stability, a strong tendency to aggregate and insolubility in non-denaturing detergents, apparently connected with a different molecular structure. All attempts to identify a post-translational chemical modification that features in the conversion of PrP^{C} into PrP^{Sc} have been unsuccessful.

The lack of a molecular explanation for the observed differences between PrP^{Sc} and PrP^{C} led to the proposal that they must differ in conformation. Indeed, Fourier transform infrared spectroscopy detected a content of 43% of β-sheet and 30% of α-helix structure for purified hamster PrP^{Sc} and

an even higher β-sheet content of 54% for PrP27-30. On the other hand a low content of β-sheet structure and a high α-helix content of 42% was found in PrP^C, suggesting differences in secondary structure between the aberrant and normal forms of PrP (Pan et al., 1993).

Due to its better solubility and the availability of recombinant forms of PrP^C, the three-dimensional structure of mouse PrP(121-231), involving three α-helices and a short antiparallel β-sheet, could be established by NMR (Riek et al. 1996). In the mature murine PrP^C(23-231), this segment seems to have the same fold (Riek et al., 1997). Also the spatial structure of recombinant hamster PrP(29-231) has been examined (Donne et al., 1997).

A species barrier for prion infection has been convincingly documented and found to vary widely depending on the pair of species involved and the direction of transmission. A structural basis for this species barrier is theoretically related to part or all of the amino acid replacements between the PrP of a given pair of species (Billeter et al., 1997).-

Within species, genetic polymorphism in the PrP gene has been found for example with mice, humans and sheep. In sheep amino acid substitutions in Prp at a few different positions were found to correlate with different predispositions for the development of scrapie (Laplanche et al., 1993; Hunter et al., 1994; Belt et al., 1995; Bossers et al., 1996).

Studies of scrapie in goats and mice demonstrated reproducible variations in disease phenotype (length of incubation times and pattern of vacuolation) with the passage of prions in genetically inbred hosts (Bruce & Fraser, 1991). The distinct varieties or isolates of prions were called "strains". Safar et al. (1998) made plausible that the biological properties of prion strains are enciphered in the conformation of PrP^{Sc} and that strains represent different

conformations of PrP^{Sc} molecules. Infection of Syrian hamsters with eight different hamster-adapted scrapie isolates produced $PrPS^{Sc}$ which, isolated from brains in the terminal stages of disease, differed with respect to protease resistance and unfolding behavior under denaturing conditions. Differences in glycosylation have also been proposed as "strain-specific" properties (Collinge et al., 1996).

Animals and humans lack a TSE disease-specific immune response and TSE diagnosis is based mainly on histopathological examination, which relies on the observation of neuronal degeneration, grey matter vacuolation (the spongiform change) and astrocytosis. A distinguishing feature of TSEs is the accumulation of aberrant protein (PrP^{Sc}) in the brain under continuing biosynthesis of the normal cellular PrP^{C}. Species differences exist however, since the accumulation of PrP^{Sc} in brains of hamster and mouse is approx. 10x as high as in the ruminant. Unlike the normal PrP^{C}, PrP^{Sc} can aggregate into amyloid-like fibrils and plaques and is a major component of brain fractions enriched for scrapie activity. Therefore, a more specific diagnosis of TSEs is detection of PrP^{Sc} either in situ e.g. by immunohistochemistry or in tissue homogenates e.g. by Western blot.

Several poly- or monoclonal antibodies to PrP have been described. The antisera were raised in mice, hamsters, rabbits and PrP null mice and as immunogens, peptides (as linear epitopes), purified and formic acid treated PrP^{Sc} from mice, hamster or sheep and recombinant PrP are being used. However, except for one case (Korth et al., 1997), there have no antibodies been developed which can discriminate between native forms of PrP^{C} and Prp^{Sc}, and such antibodies cannot likely discern the difference between prion strains.

By Western blotting or immunohistochemistry PrP^{Sc} could be detected in sheep in brain, spleen, tonsil or lymph node material and even in a preclinical stage of scrapie (Schreuder et al., 1998).

The intriguing mechanism of prion replication is not fully understood. According to the prevailing theory, the infectious PrP^{Sc} acts as a template in the replication of nascent PrP^{Sc} molecules. In other words PrP^{Sc} imposes its own conformation upon the cellular form PrP^{C} or an intermediate form. A thus far unknown protein X may function as a molecular chaperone in this formation of PrP^{Sc} (Prusiner et al., 1998).

Because of the connection between BSE and the nvCJD, and the possible transfer of BSE to other species including sheep, there is a need to monitor slaughter cattle and sheep for the presence of aberrant prion protein before the meat and meat products enter the human and animal food chain or into pharmaceuticals prepared for human and animal use. Mass screening of sheep and cattle should also be of help in view of eradication programmes of scrapie and BSE. Moreover, human blood and blood products may form a health threat on account of possible contamination with blood of CJD patients and the recent occurrence of the nvCJD. For these monitoring purposes a detection method for aberrant prion protein has to be developed which should be both fast, sensitive, reliable and simple.

Bioassays for PrP^{Sc} in which different doses of the analyte are administered to target animals, are generally regarded a gold standard but otherwise are cumbersome and costly. Moreover, their quantitative character is limited by a high variation. Immunohistochemical (IHC) approaches are very useful insofar the presence of the analyte is directly made visible in the infected tissue. Also the presence of

PrP^{Sc} can be indicated in a preclinical phase. However, these methods are not quantitative, and hardly applicable on a large scale.

For the diagnosis of TSEs founded on the demonstration of PrP^{Sc} in infected tissues and for the assessment of PrP^{Sc} itself, several methods have been described and all are on an immunochemical basis. Most of these tests have been developed and used for research-like purposes, for instance in order to quantify PrP^{Sc} during purification procedures. In some cases calibration was with recombinant PrP (hamster or mice) or with PrP^{Sc}, purified from scrapie-infected brains. Otherwise, responses were expressed as a function of mg tissue equivalents; in this way also sensitivity could be assessed by the minimum amount of tissue required for the PrP^{Sc} detection.

ELISA systems were designed for detection of PrP^{Sc}, isolated from brains of scrapie-affected mice and hamsters (Kascsak et al, 1987) and PrP^{Sc} from murine brain and spleen (Grathwohl et al., 1997). In these assays, the PrP^{C} fraction was beforehand removed by PK-treatment and the purified and solubilised analyte was directly coated onto the microtiter plate. Solubilisation of PrP^{Sc} was by treatment with SDS or extraction with 77% formic acid, drying and resuspension in buffer (Kascsak et al. 1987). The denaturing action of formic acid was found to enhance the antibody response to PrP^{Sc} considerably compared to untreatred or SDS-treated material. In this ELISA rabbit antiserum to the mouse scrapie strain ME7 PrP^{Sc} was used.

Also successive solubilization of purified PrP^{Sc} by boiling in SDS, precipitation in cold methanol and sonication in 3-4 M guanidine thiocyanate (gdnSCN) (Grathwohl et al., 1997) apparently enhanced coating-efficiency and/or epitope density under the denaturing action of gdnSCN. On the other hand, dissolving PrP^{Sc} in SDS appeared to inhibit adsorption

of PrP^{Sc} onto the polystyrene microtiter plate. Although Grathwohl et al. (1997) state that their method could be a basis for a sensitive screening method for PrP^{Sc} in crude tissue extracts, their extraction and purification steps are impracticable and time-consuming (over 22 h). The sensitivity for brain tissue was such that PrP^{Sc} could be detected in 39 mg brain equivalents; the corresponding figure for spleen tissue amounted to 313 mg.

A sandwich type of ELISA was used to monitor the bioproduction of recombinant hamster PrP(90-231), the protease resistant core of PrP^{Sc} (Mehlhorn et al., 1996). As a capture antibody the Fab fragment of mAb 3F4 was coated. This antibody was raised against hamster scrapie strain 263K and reacts with hamster, human and feline PrP. As the second antibody mAb 13A5 (to scrapie hamster PrP^{Sc}) was used. Samples from the different stages of purification were measured in this ELISA. However, the practical conditions under which PrP^{Sc}, in order to be detected as an antigen, is brought into an unfolded state by chaotropic agents like 3-4 M gdnSCN, are not to be combined well with the format of a sandwich type of ELISA.

Prusiner et al. (1990) used an enzyme-linked immunofiltration assay (ELIFA) which combines the properties of an immuno-dot blot and ELISA technique. By this method both PrP^{C} and PrP^{Sc} in scrapie brain homogenates of hamsters could be quantified against a standard curve of known amounts of purified hamster PrP27-30 (0.06-4 ng). Brain homogenates, diluted in buffer with 1 M gdnSCN and 0.05% Tween 20, were applied in 5 µl quantities to nitrocellulose membrane in a manifold filtration unit. Sequential steps for immunocomplex formation with mAb 13A5 and conjugation of enzyme were also done on this membrane. For detection, dots were cut out with a puncher and placed into a microtiter plate in which color was developed. Under these conditions, immunoreactivity of

the dissociated and (partly) unfolded PrP^{Sc} is indistinguishable from that of PrP^{C} and in this way total PrP was measured. For the determination of the PrP^{Sc} fraction, the homogenate was treated with PK prior to the ELISA and PrP^{C} content was calculated by subtracting the PrP^{Sc} from the total PrP.

Oesch et al.(1994) refined this ELIFA method. Samples were applied on nitrocellulose filters in the ELIFA apparatus, procedures hereafter among which a 2h-preincubation in 4 M gdnSCN and substrate binding to mAb13A5, up to and including binding with the enzyme were done on the membrane taken out of the apparatus. For detection, membranes were placed back in the ELIFA apparatus and reacted with substrate solution. Finally, the reaction mixture was pulled through into an ELISA plate placed underneath and colour development was measured. This whole procedure took over 20 hours.

Immuno-dot blotting was used by Serban et al. (1990) for the post mortem diagnosis of Creutzfeldt-Jakob disease in humans, scrapie in sheep and scrapie-infected hamsters and mice. Direct spotting of a rather impure analyte on e.g. nitrocellulose filters instead of adsorption of a purified fraction of it onto the plastic surface of microtiter wells produces a more robust ELISA variant. This qualitative test was based on the intensified immunoreactivity of PrP^{Sc}-containing amyloid plaques after treatment with 3 M gdnSCN and the protease resistance of the PrP^{Sc} isoform.

Brains were extracted in detergent-containing lysis buffer and 4 µl amounts were spotted onto nitrocellulose membranes. Immunoreactivity of the spotted material after successive treatment with PK and 3 M gdnSCN was conclusive for the presence of PrP^{Sc} and confirmation of CJD and scrapie. Rabbit Ab R075 (to purified hamster PrP27-30) was able to detect PrP in the above four species. Out of a total

of 28 human brain samples, 9 cases found positive by this method were also either defined as CJD or GSS by both clinical diagnosis and a histopathological examination. For two cases, found positive by the blot procedure, histopathologic results were not available. The negative results of histopathology for CJD or GSS on the remaining 17 cases, coincided also with no indication for PrP^{Sc} with the immuno-dot blot assay. In 12 histologically confirmed cases of natural scrapie in sheep, PrP^{Sc} was detected with the immunoblotting technique in the brains of 11 sheep. There are variations in the distribution of PrP^{Sc} in the brain of scrapie-affected sheep, since PrP^{Sc} was found in the spinal cord, cerebellum and pons/medulla of 2 sheep, but one sheep also had PrP^{Sc} in the frontal and occipital cortex and the thalamus. This means that sampling of brain tissue could lead to false negatives due to regional variations in PrP^{Sc} content. The detection limit of this method for brain extracts of scrapie-infected hamsters and mice ranged from 5-132 mg tissue equivalents, because these amounts still gave clearly visible spots. The duration of the test was, apart from an overnight incubation step, 6h.

Safar et al. (1998) developed a conformation-dependant fluorescent-ELISA that can discern various prion strains of hamsters. The assay detects a region of PrP^{Sc} that while exposed in normal PrP^{C}, becomes folded in the PrP^{Sc} molecule. Eu-labeled mAb 3F4 that reacts with a region of PrP^{Sc} only after unfolding in 4 M gdnHCl and heating at 80°C for 5 min, was used in this assay. The immunoreactivity of the antibody to the denaturated region, as reflected by the fluorescence signal, is much higher than it is to PrP^{Sc} in its native form. The authors developed an algorithm which takes into account that the immunoreactivity of antibody to denatured PrP in a sample of an affected brain is the summation of enhanced immunoreactivities of PrP^{Sc} and PrP^{C} during the

transition from the native to the denatured states. Knowledge of the enhancement of immunoreactivity for PrP^C during denaturation was a prerequisite for this approach. For this purpose calibration curves with different concentrations of purified PrP^C were constructed. It appeared that also PrP^C showed an enhanced immunoreactivity in 4 M gdnHCl compared to its native state, albeit in a moderate way (≤ 1.8x). From the algorithm and the measurements of a native as well as a denatured sample, the content of PrP^{Sc} could be calculated. Although this method was validated for the determination of hamster brain, the authors aim at using it also for the detection of other mammalian prions, including human. In order to improve the detection threshold of the assay they introduced an initial step to selectively precipitate PrP^{Sc} from raw material with sodium phosphotungstate. In combination with this sample pretreatment, the final sensitivity of the assay could be made high. The sensitivity limit is less than or equal to 1 ng/ml (100 pg) of PrP^{Sc}. The test however, is still far from lending itself to large-scale use in view of too much labour and long incubation times.

Capillary electrophoresis was adapted by Schmerr et al. (1995, 1996, 1998a) as a diagnostic, immunochemical assay for scrapie. The authors claim a high sensitivity (approx. 135 pg PrP^{Sc}) of their test by measuring laser-induced fluorescence of a PrP-derived fluorescein-labeled peptide after its separation by free zone capillary electrophoresis. In a preceding competition step, this peptide was displaced from a preformed complex of the peptide and an antibody directed to the unlabeled peptide in competition with the analyte (PrP^{Sc}). Beforehand, PrP^C had been removed from the analyte solution by PK-treatment. The concentration of rabbit antiserum for complex-preformation was chosen so that the antibody would be limiting in the assay (adjustment to 50% of the maximum amount of immunocomplex). Four anti-(prion)-

peptide antisera were prepared and evaluated. Assays using antisera to the peptides spanning mouse amino acid position 142-154 and 155-178, differentiated scrapie-positive sheep from normal animals. In spite of the high sensitivity of this method, sample processing is time-consuming (approx. 24h) and cumbersome since Prp^{Sc} from brain stem has to be concentrated and purified through steps like ultracentrifugation and HPLC.

Western blotting (WB) in combination with SDS-PAGE is also a suitable technique for diagnosis of TSEs and a variety of different extraction procedures and Western blotting methods has been described (Race et al., 1992; Beekes et al., 1995).

Usually, PrP^{C} is extracted from tissues with detergents that solubilize this membrane-bound protein in a mixed micelle. However, PrP^{Sc} in the presence of detergents, aggregates and therefore is not solubilized but can be spun down by ultracentrifugation. PrP^{Sc}-aggregates dissociate in monomers under the denaturing conditions of heating in SDS solution with β-mercaptoethanol. In this way PrP^{Sc} is electrophoretically (SDS-PAGE) indistinguishable from PrP^{C}, unless a preceding treatment with PK has been applied. This proteolytic treatment removes PrP^{C} and leaves PrP27-30, the truncated form of PrP^{Sc}.

Race et al. (1992) could find PrP^{Sc} in every brain of 8 sheep that were histologically positive for scrapie and even in brains of clinically positive sheep that were not diagnosed as scrapie-positive by histology. For detection anti-peptide antibodies to residues 89-103 and 218-232 of the mouse PrP sequence were used. Apparently, the amount of tissue required to visualise PrP^{Sc} varied among sheep from <2 to 200 mg equivalents of brain tissue. Also PrP^{Sc} was found in spleens and lymph nodes in 7 of 8 sheep that had the protease-resistant form detected in brain homogenates.

One method based on WB was officially approved by the European Union (EU) and the World Organisation for Animal Health (OIE) for BSE and scrapie diagnosis (Bradley et al., 1994). A minimum amount of 2 mg equivalent of infected scrapie brain allows detection of the PrP27-30.

Above identified assays have never been used in large screening efforts for the detection of aberrant prion protein neither in animals nor in humans.

Thus far, two commercial assays have been announced. In 1997 the Swiss company Prionics Inc. launched its "BSE Western Test" intended for mass screening of slaughter cattle. A modified and optimised Western blot method was used to detect the proteinase K-resistant PrP27-30 in bovine brain stem. For immunodetection mAb 6H4 was used, developed by immunizing PrP-null mice with recombinant bovine PrP. This antibody recognizes residues 147-155 of the bovine sequence as a linear epitope in native PrP^{C} and denatured PrP^{Sc}; this sequence is also recognised in sheep, human, pig and mouse. Incubation with anti-mouse IgG coupled to alkaline phosphatase and detection of the enzymatic product by chemiluminescence were the final steps of the assay. This test requires an incubation step with PK and detects PrP27-30. Reliability is strengthened by the Western blot documentation of the decrease in size (internal control) of the prion protein from 30-33 to 27-30 kDa. The test can be done within hours and the expectation is that subclinical BSE in post-mortem brains may be detected.

Also in 1997 the Irish Company Enfer Scientific Ltd. announced the development of a BSE post-mortem test. This immunoassay intended for mass screening uses a PrP antipeptide antiserum to detect PrP27-30 in samples of brain tissue of cattle after removal of PrP^{C} by PK-treatment. Immunodetection was enhanced by chemiluminescence. Their

claims are a result within 4 hours after receipt of samples and a capacity of 14,000 cattle a day and moreover, the catching of asymptomatic animals.

However, these two commercial tests, although claiming high sensitivity in detecting the aberrant protein, and thus claiming to have a low number of false-negative results, suffer from the low specificity associated with the claimed high sensitivity. When using the above tests one therefore runs an increased risk of falsely identifying a negative sample as false-positive, thereby falsely identifying an animal as positive. For example, Switzerland slaughtered herds in which one or more cases of BSE had been confirmed. The "Swiss reference laboratory for animal TSE" examined the brains of these 1761 apparently healthy cattle by an immunohistochemical method for signs of BSE and six positive cases were detected. Also Prionics Inc. tested these 1761 cattle brains by their "BSE Western Test". Four positive outcomes were identical to the ones found by the reference laboratory, the other two were indicated as negative and moreover two other cattle were found positive by Western blotting. Thus a total of eight positive reactors were found, four of which overlapped. These eight were re-examined in the laboratory of Dr Kretzschmar (University of Göttingen) and in addition to the four undisputed cases, one of the two questionable cases identified by the reference laboratory could be confirmed (info: New Scientist, 1998, July 4 and Internet). Prionics for example scored 0.1% false-positives, indicating that in 1 of every thousand cases a sample causes a false-alarm due to false-positivity.

Tests scoring false-positive results (being in general not specific enough) have other consequences than tests scoring false-negative results (being in general not sensitive enough).

False-negative means that an in essence positive sample from a positive individual is scored negative, and thus is not suspected of having a TSE while in truth said individual is having a TSE. A false-negative diagnosis thus results in missing positive cases.

For humans, false-negative means that no diagnosis of TSE is made where said human actually has a TSE. This causes a wrong prognosis being established and wrong treatment being given, until a second test is done.

For animals, especially in those cases where slaughtered animals are tested, false-negative means that no diagnosis of TSE is made where said animal was actually infected and possibly capable of spreading the disease without having been noticed. Meat and other products from such a false-negative animal may contain aberrant prion protein. Such meat and meat products will be traded and eaten, and can thus be a source for further infection, notably of humans who even falsely trust that the animal has been tested well and the meat or meat product bears no risk.

False-positive means that an in essence negative sample from a negative individual is scored positive, and thus is at least suspected of having a TSE while in truth said individual is not having a TSE at all, but possibly another condition.

For humans, false-positive means that a false diagnosis of TSE is made, here again resulting in false prognosis, and in faulty treatment. If said individual is not treated well as a consequence of the mis-diagnosis, his or her possible other disease condition (the symptoms of which for example gave rise to the decision to test for TSE) receives no proper treatment.

For animals, false-positive means that a false diagnosis of TSE is made, however, since TSEs are notifiable diseases that in general are met with strict eradication measures,

said animal shall, at least in most Western countries be killed and destroyed. Furthermore, the herd from which said animals originated runs the same risk of being destroyed when the diagnosis is not corrected. For the slaughterhouse it might mean that special laborious decontamination actions have to be implemented which mean temporary interference of use of the facilities and thus considerable loss of productivity. Additionally, the country where said animal or herd is falsely diagnosed for having a case of TSE among its animals will be met with export restrictions. It goes without saying that, especially when said country has no (present) reported cases of TSE, such a false-positive diagnosis is highly detrimental for said countries position on foreign markets for animal products.

Understanding the above risks associated with false-negative or false-positive diagnoses becomes even more complicated when one understands that in general the level of false-positives scored by a diagnostic method or test is inversely related to the number of false-negatives scored by the same test. It is an old diagnostic truth that, in many instances, a very sensitive test (having low numbers of false-negatives) cannot be very specific (and thus has a relative high number of false-positives) and vice versa. However, and especially for mass screening tests wherein many samples need to be tested, tests having both high sensitivity and specificity are desired.

The invention provides use of guanidine thiocyanate (gdnSCN) or a functional equivalent thereof for treating at least one sample derived from a mammal for reducing the risk of scoring a false-positive test result in testing said sample for the presence or absence of aberrant prion protein. Using guanidine thiocyanate or its functional equivalents allows reduction of the signal arising from the normal prion

protein (PrP^c) so that, for example when a gdnSCN-treated sample is compared with an untreated sample, the PrP^c signal is greatly reduced. See for example Figure 3 herein describing the reduction of the signal from a PrP^{Sc} negative sample obtained by a use according to the invention. In a preferred embodiment, the invention provides use of guanidine thiocyanate (gdnSCN) or a functional equivalent thereof for treating at least one sample derived from a mammal for reducing the risks of scoring both a false-positive test result or a false-negative test result in testing samples for the presence or absence of aberrant prion protein. In a preferred embodiment, the invention provides use according to the invention in an immunoassay. The invention provides a reliable, simple and fast method, comprising use of gdnSCN or a functional equivalent thereof in a method for diagnosis of TSE being both highly specific as well as highly sensitive.

The invention also provides a method for reducing the risk of scoring a false-positive test result in testing a sample derived from a mammal for the presence or absence of aberrant prion protein comprising treating at least one sample with gdnSCN or a functional equivalent thereof. Because of its simplicity and speed this method particularly lends itself to mass screening purposes of e.g. post-mortem tissues in the slaughter-line of ruminants such as cattle and sheep, but it is equally suitable in testing samples derived from other ruminants or experimental animals. In the human field the method could be used for e.g. screening lymphoid tissues and blood-derived products. Essentially, samples from all tissues, body fluids (e.g. blood, liquor) and faeces can be used.

BSE or subclinical (silent) cases of BSE can for example be detected in samples automatically taken from the brain at the time that the heads are cut off from the slaughter-animals' trunk. The method can also be used in preclinical

stages during the development of scrapie, since tonsils which can be taken from the living animal, are proven to be an indicator tissue for preclinical scrapie and to contain PrP^{Sc} (Schreuder et al., 1998).

With scrapie in sheep as a model, we developed a method to unambiguously distinguish PrP^{Sc} from PrP^{C}. This can be done on the basis of immunodetection of PrP^{Sc} without the need of a preceding elimination of PrP^{C} by enzymatic proteolysis (Figure 3).

In a modified embodiment, the invention provides use according to the invention comprising use of a protease for treating said sample to reduce the presence of normal prion protein (Figure 4). This design is for example suited for the detection of aberrant prion protein in BSE.

Immunologically the signal of PrP^{Sc} can be enhanced in the presence of chaotropic agents. This enhancement is undoubtedly effected by dissociation of the polymeric PrP^{Sc} into oligo- or monomeric units, resulting in an increase of the number of epitopes available for the antibody. Moreover, the epitopes may be better exposed to the antibody through the protein defolding action of the denaturant.

Furthermore, the invention provides a method further comprising treating at least one first sample with gdnSCN or a functional equivalent thereof and leaving at least one second sample untreated with gdnSCN or a functional equivalent thereof and comparing the test result of said first sample with said second sample. We can for example discriminate between TSE-positive and negative cases after duplicate dot blotting of extracts of brain tissue onto nitrocellulose membrane. Extraction is in detergent-containing (lysis) buffer. One aliquot is left untreated (U) and the other one is treated in 4 M gdnSCN (Figure 3) or, after PK-digestion, treated in 4 M gdnSCN (Figure 4). Then, after immunostaining, the treated sample is compared to the

untreated sample, a higher signal means: TSE-positive and a lower signal: TSE-negative. Numerically, any value ≥1 for the ratio of intensities I_T/I_U means TSE-positive and values <<1, TSE-negative. By this dual internal control the discriminating value of the test is considerably enforced.

The invention also provides a method according to the invention comprising immunological detection of said aberrant prion protein using at least one antibody directed against a proteinase K resistant part of the aberrant prion protein, for example wherein said antibody is directed against a proteinase K resistant N-terminal part of the aberrant prion protein. In a preferred antibody said antibody is raised against a peptide derived from the prion protein, for example wherein said peptide is selected from an N-terminal group consisting of residues 94-111 (like 94-105 and 100-111), a C-terminal group consisting of residues 222-234 and a group consisting of residues 145-177 (sheep numbering) or sequential homologs of the PK-resistant part of PrP^{Sc} (Figure 2) or functional equivalents thereof.

The invention also provides a method according to the invention wherein said protein is immunologically detected in an enzyme-linked immunoassay, for example wherein said enzyme-linked immunoassay comprises a dot-blot assay.

Also, the invention provides a test kit having been provided with means for performing a method according to the invention. This kit for example contains a carrier matrix for spotting sample extracts from tissues, organs, cells or body fluids (e.g. nerve-tissue, blood cells, etc), buffers, solutions of gdnSCN and PK, primary antibody, enzyme-labeled second antibody and enzyme-substrate. In a most preferred embodiment, said method or test kit is designed for mass-screening purposes.

The invention is further described in the detailed description herein without limiting the invention.

FURTHER DETAILED DESCRIPTION

MATERIALS AND METHODS

Phosphate buffered saline (PBS), pH 7.2 contained 136.89 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4 and 2.79 mM KH_2PO_4 in water.

PBTS: 0.2% (w/v) Tween-20 in PBS.

Two extraction buffers were used:

(a) 10 mM phosphate buffer, pH 7.0, 0.15 M NaCl and 0.25 M sucrose, used by Pan et al. (1992) to prepare microsomal fractions;

(b) lysis buffer (Collinge et al., 1996) consisted of 0.5% (w/v) Tergitol (type NP-40, nonylphenoxy polyethoxy ethanol, Sigma NP-40) and 0.5% (w/v) deoxycholic acid, Na-salt (Merck) in PBS, pH 7.20.

Guanidine thiocyanate (gdnSCN, purity > 99%; Sigma G 9277) solutions of 4 M were made up in water (pH 5.8).

Alkaline phosphatase-conjugated goat anti-rabbit IgG (GAR/AP) was from Southern Biotechnology Ass. (ITK, Diagnostics B.V., Uithoorn).

Substrate for alkaline phosphatase was 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; tablets; Sigma B5655).

Usually, after PrP extraction, protease inhibitors were added to the extracts.(Complete, protease inhibitor cocktail tablets; Boehringer Nr. 1697498, Mannheim, Germany).

Proteinase K (EC 3.4.21.14, 20 units/mg lyophilisate

Nr. 745723) and Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride Nr. 1585916) were also from Boehringer. Incubation conditions for PrP-extracts with PK were 50 µg/ml enzyme for 30 min at 37°C. In order to stop this enzymatic reaction, the incubation mixture was made 1 mM in Pefabloc added from a 100 mM stock solution of the inhibitor in water.

As a blocking agent nonfat dry milk (Protifar, Nutricia) was used.

Three membrane types were used: nitrocellulose (NC) membrane with a 3 mm screen (Protran BA 85/21; 0.45 mm Nr. 405891) was from Schleicher & Schuell GmbH (Dassel, Germany), Immobilon-P, (polyvinylidenedifluoride, PVDF) from Millipore B.V. (Etten Leur) and Zeta-Probe (quaternary amine-nylon membrane) was from BioRad.

An Ultra-Turrax T25 mixer with a 10 mm shaft (IkA Labortechnik Gmbh, Staufen, Germany) was used to homogenize brain tissue. The shaft was decontaminated in 1 M NaOH.

Water of 'Milli-Q' (Millipore) quality was used throughout.

Primary antibodies

These were intentionally designed for scrapie diagnosis. Antisera were induced in rabbits using synthetic peptides with sequences (12-mers) based on the sequence of ovine PrP protein. The sequences have such differences with the rabbit PrP sequence that they induce not only antibodies which recognise these peptides but also the authentic PrP protein. Other animal species like mouse which have sequence differences could be as well suitable. The sequences used for immunisation were selected 12-mers from the protease K-resistant domain of PrP^{Sc}. The selected 12-mer sequences

represent domains that have a low tendency to form secondary structure (α-helix or β-sheet). The antisera are reactive in diagnostic dot blotting but also in Western blotting of both PrP^{C} and PrP^{Sc}, in ELISAs with as coated antigens the above, peptides or PrP protein, and in immunohistochemical detection. With the peptide derived from the ovine prion protein sequence 94-105, antisera R521 and R522 were produced in rabbits. Likewise sequence 100-111 yielded antisera R504, R505, R593, R594, R595, R596 and sequence 145-177 antiserum R532. The sequence 126-143 (ovine and bovine) gave rise to antiserum R568 while sequence 223-234 (ovine and bovine) yielded antisera R523 and R524. Peptides were synthesized and used to raise anti-peptide antisera in rabbits following previously published procedures (Van Keulen et al., 1995). Antisera were confirmed to be specific for sheep PrP (both undigested and after proteinase K treatment) on Western blots of partially purified prion protein from scrapie-affected sheep brain.

Sheep samples (brain stem, cervical spinal cord) were from scrapie-affected sheep, diagnosed by histopathological and immunohistochemical examination of the brain and from normal healthy sheep (Van Keulen et al., 1995). Samples from BSE-diagnosed cattle (histopathology, immunohistochemical examination and Western blotting) were from the cervical spinal cord.

Procedure for immuno-dot blotting: 0.5 g portions of brain tissue were cut down with a scalpel and homogenized with an Ultra-Turrax mixer (20.000 rpm/15 sec) in 4.5 ml of ice-cold lysis buffer. The homogenates were centrifuged at 1000xg for 10 min. If appropriate, an aliquot of the homogenate was incubated with PK at 37°C for 30 min after which the reaction was stopped with Pefabloc (1 mM). Otherwise a cocktail of protease inhibitors was immediately added to the homogenate.

Suitable dilutions of the turbid supernatants in lysis buffer were spotted in 1-3 µl amounts onto two blotting membranes and left for drying (15 min). One membrane was incubated in 4 M gdnSCN for 10 min, the other membrane was left untreated. Washing of the membranes was for 10 min in PBS on a rocking platform.

Membranes were blocked with 5% (w/v) Protifar in PBS for 1h at 20 °C and washed in PBTS for 5 min at 20°C. A 2h incubation with the primary antibody (1/1000 diluted in PBTS) at 20°C was followed by three washing steps in PBTS for 5 min each. Next, the membranes were incubated with AP-conjugated goat anti-rabbit IgG (1/1000 diluted in PBTS) for 2 h at 20°C and washed in PBTS three times for 5 min. Substrate solution was added and the reaction was stopped with water.

RESULTS

DETECTION OF ABERRANT PRION PROTEIN IN SCRAPIE

Extraction efficiency for PrP^{Sc}

After homogenizing brain stem tissue of a scrapie-affected sheep in extraction buffer (a) or in (b) (= lysis buffer) and low-speed centrifugation which yielded supernatant 1, aliquots of this supernatant were again centrifuged at a higher speed (11,000xg, 10 min: 'high speed' supernatant 1). The loose pellets left from the first centrifugation step were adjusted with buffer to the original volume, re-extracted and centrifuged at 1000xg, which yielded a supernatant 2 and a loose pellet. In addition, aliquots of all fractions were treated with PK.

1 µl extracts (diluted 1, 1/10 and 1/100x in their respective buffers) were spotted onto NC and immunodetection was with R522-7, an antiserum that has proven to detect ovine PrP (Van Keulen et al, 1995).

For lysis buffer the highest signal intensity was obtained for the supernatant 1. Compared to the results for lysis buffer, the signals for extraction buffer (a) were lower for all fractions, except for the pellet. For fractions of the lysis buffer, decreased intensities were observed after pretreatment with proteinase K, especially for supernatant 2, which indicates that this fraction is relatively enriched with PrP^C.

We observed dramatically intensified signals for the lysis buffer extracts, when these were diluted in 4 M gdnSCN. For supernatant 1, even after a 100-fold dilution, the signal was clearly visible, which means that these scrapie brain stems PrP can be made visible in a tissue equivalent of 1 µg.

Divergent signal enhancement for PrP^{Sc} and PrP^C

Investigation of brain stem extracts of a scrapie-negative sheep revealed, even in a 80-fold dilution, clear signals of PrP^C. However, after pretreatment which PK no signal could anymore be observed. Surprisingly, instead of applying this PK-treatment, dilution of tissue extract in 4 M gdnSCN led also to a dramatic decrease of signal intensity for PrP^C.

Next, instead of diluting lysis buffer extracted samples in 4 M gdnSCN, we applied serial dilutions of brain extracts of scrapie-positive and negatieve sheep in duplicate on NC membranes and incubated one membrane in 4 M gdnSCN for 10 min while the other one was left untreated.

Immunodetection revealed that we easily could discriminate between scrapie positive (PrP^{Sc} and PrP^C) and scrapie negative (PrP^C) samples: a higher intensity with 4 M gdnSCN compared to an untreated sample means scrapie positive, while a lower intensity with gdnSCN means scrapie negative.

This finding is the basis for a rapid and simple diagnostic test for TSEs. In this test there is in general no need for a preceding removal of PrP^C from the negative sample.

Alternative denaturants and antisera

As an alternative for gdnSCN we investigated the effects of other chaotropic agents. After dot blotting 3 µl dilutions of extracts of scrapie positive and negative brain stems, separate NC membranes were incubated for 10 min in chaotropic agents. The solutions used were: 4 M gdnSCN, 7.2 M urea, 4 M KSCN, 1 M thiourea, NaOH (pH 11) in water and 98% formic acid; besides one membrane was left untreated as a blank. Results for immunodetection after KSCN and thiourea did not differ from the blank. Urea induced a slight increase for the scrapie positive material and formic acid enhanced the intensity to the level of gdnSCN although this acid caused considerable shrinking of the NC membrane. NaOH (pH 11) on the other hand increased the signal for scrapie-negative material.

Treatment with 4 M gdnSCN turned out to be the best discrimination between scrapie positive and negative tissue samples. Moreover, this effect appeared to be pH-invariant since solutions of 4 M gdnSCN at pH 4 and 7 (in 50 mM phosphate buffer), pH 6 (in water) and pH 9 (in 50 mM carbonate buffer) gave identical results.

Five classes of antipeptide antisera to linear epitopes of sheep PrP sequences (94-105, 100-111, 126-143, 145-177 and 223-234) were examined. For comparative reasons all sera were used in a 1/500 dilution in PBTS. Antisera to the 94-105 sequence (R521, R522) and to the 100-111 sequence (R 505) proved to have the best differentiating power. On the other hand, with the antisera R568 and R532 to the sequences 126-143 and 145-177 respectively, no immunoenhancing effect of

4 M gdnSCN on PrP^{Sc} could be detected.

Blotting membranes.

Comparison of results on NC membrane with those on Zeta-Probe showed for the latter a strong aspecific coloring of the entire membrane and consequently quaternary amine-nylon as a carrier was unsuitable. On the other hand, compared to nitrocellulose a stronger adsorption for PrP was shown for the PVDF membrane (Immobilon-P).

DETECTION OF ABERRANT PRION PROTEIN IN BSE

From brains of BSE-positive cattle, obtained from The Netherlands, the UK and Belgium and of Dutch BSE-negative cattle (diagnosed by histopathology and immunohistochemical examination), brain stems were extracted with lysis buffer in the same manner as for sheep and the low-speed supernatant 1 was used for further examination. Brain stem extracts from confirmed scrapie-negative and positive sheep were used for comparison. Aliquots of extracts were also treated with proteinase K and 3 µl amounts of dilutions in lysis buffer of PK-treated and untreated extracts were spotted onto NC membranes. Immunodetection was with 1/1000 dilutions of antisera to the 12-mer sequences 94-105 (antiserum R521), 100-111 (R505, R595, R596), 223-234 (R523, R524) and to the longer sequences 126-143 (R568) and 145-177 (R532). Highest immunoreactivity was shown with antisera R505 and R595. After incubation with 4 M gdnSCN signal intensity of BSE-negative samples diminished; however, the immunoenhancing effect of 4 M gdnSCN on PrP^{Sc} in BSE-positive samples did not reach a comparable high level as for sheep PrP^{Sc} in scrapie. Surprisingly, antisera R523, R524 and especially R532 showed stronger immunoreactivity with bovine PrP^{Sc} than with PrP^{C}. Immunoreactivity of antisera R521 and R568 with bovine PrP

was very poor. No signal was obtained with the PK-treated material of BSE- and scrapie negative animals. PVDF showed a higher adsorption than NC membranes since immunostaining could be observed at higher dilutions on PVDF.

The design of one of our tests is that of a dot-blot immunoassay which has an intrinsically higher sensitivity than an analogous ELISA assay in a microtiter plate, due to miniaturization within the blot and the higher binding capacity of the matrix material (nitrocellulose, PVDF) than of a smooth polysterene microtiter plate surface. Because of the divergent immunoreactivity of sheep PrP^{C} and PrP^{Sc} during denaturation, the discriminatory power for false positive samples of our test is much higher than that of the assay of Safar and coworkers: in our assay the signal for PrP^{C} during denaturation in 4 M gdnSCN diminishes, whereas immunoenhancement (with 4 M gdnHCl) takes place in the assay of Safar and coworkers. As distinct from the test of Safar and coworkers, there is no need to calibrate our assay, it can be performed within four hours and it lends itself to automation. Quantification will be with densitometric techniques. Other options for the design of our assay are an ELIFA-format combined with detection in solution of an enzyme-enhanced fluorescence or luminescence signal or time-resolved detection of lanthanide fluorescence.

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LEGENDS TO THE FIGURES

Figure 1:
Amino acid sequences of human, rabbit, hamster, mouse, cattle and sheep PrP genes. The entire amino acid sequence of human PrP is given; open spaces in the other sequences indicate identity. Polymorphisms are indicated in bold at the top of each block and relate to the shaded positions. ↓: PHGGGWGQ. |: protease-sensitive site, right of which the sequence for the PK-resistant core of PrP^{SC} is found.

human:

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Figure 2:
Peptide sequences, derived from the prion protein structures of six species (***hu***=human, ***rb***=rabbit, ***ha***=hamster, ***mo***=mouse, ***bo***=cattle, ***ov***=sheep). The amino acid sequence of the human peptides is given; open spaces in the other sequences indicate identity.
Antipeptide antibodies were raised in rabbits against the peptides of the ovine structure. Corresponding antisera are indicated ***R5xx*** at the top of each set of sequences.

Figure 3:
Prion test in which the extract was applied to two pieces of NC-membrane indicated: untreated and treated. A 10% (wt/vol.%) extract of brain-stem tissue was 1/3 diluted and

1 µl applied to NC-membrane. Then, one piece of membrane was incubated in solution without gdnSCN (untreated), the other was incubated in 4 M gdnSCN-containing solution (treated). Further incubations for immunochemical visualization with first antibody (R522-7) and alkaline-phosphatase conjugate were according to standard procedures.
In each 3-fold dilution series, the first (left) spot represents 33 µg of tissue equivalents.

Figure 4:
Prion test, in which the extract was applied to two pieces of PVDF-membrane indicated: untreated and treated. For each negative and positive case, a 10% (wt/vol.%) extract of brain-stem tissue was prepared and divided in two portions, of which one was incubated with proteinase K (PK-digested extract) or not (undigested extract). Next, each of the extracts was 1/3 diluted and 3 µl applied to PVDF-membrane. Then, the piece of membrane with the undigested extract was incubated in solution without gdnSCN (untreated), the membrane with digested extract was incubated in 4 M gdnSCN-containing solution (treated). Further incubations for immunochemical visualization with first antibody (R595-4) and alkaline-phosphatase conjugate were according to standard procedures.
In each 3-fold dilution series, the first (left) spot represents 100 µg of tissue equivalents.

Claims

1. Use of guanidine thiocyanate (gdnSCN) or a functional equivalent thereof for treating at least one sample derived from a mammal for reducing the risk of scoring a false-positive test result in testing said sample for the presence or absence of aberrant prion protein.

2. Use according to claim 1 for reducing the risk of scoring a false-negative test result.

3. Use according to claim 1 or 2 wherein said sample is tested in a immunoassay.

4. Use according to anyone of claims 1 to 3 wherein said immunoassay is designed for mass-screening purposes.

5. Use according to anyone of claims 1 to 4 further comprising use of a protease for treating said sample to reduce the presence of normal prion protein.

6. Use according to anyone of claims 1 to 5 wherein said sample is derived from a ruminant.

7. Use according to claim 6 wherein said ruminant is ovine or bovine.

8. A method for reducing the risk of scoring a false-positive test result in testing a sample derived from a mammal for the presence or absence of aberrant prion protein comprising treating at least one sample with gdnSCN or a functional equivalent thereof.

9. A method according to claim 8 further comprising reducing the risk of scoring a false-negative result.

10. A method according to claim 8 or 9 further comprising treating at least one first sample with gdnSCN or a functional equivalent thereof and leaving at least one second sample untreated with gdnSCN or a functional equivalent thereof and comparing the test result of said first sample with said second sample.

11. A method according to anyone of claims 8 to 10 further comprising immunological detection of said aberrant prion protein using at least one antibody directed against a proteinase K resistant part of the aberrant prion protein.

12. A method according to claim 11 wherein said antibody is directed against a proteinase K resistant N-terminal part of the aberrant prion protein.

13. A method according to claim 11 or 12 wherein said antibody is raised against a peptide derived from the prion protein.

14. A method according to claim 13 wherein said peptide is selected from the groups listed in figure 2 or functional equivalents thereof.

15. A method according to anyone of claims 11 to 14 wherein said protein is immunologically detected in an enzyme-linked immunoassay.

16. A method according to claim 15 wherein said enzyme-linked immunoassay comprises a dot-blot assay.

17. A method according to any one of claims 8 to 16 wherein said mammal is ruminant, preferably wherein said ruminant is ovine or bovine.

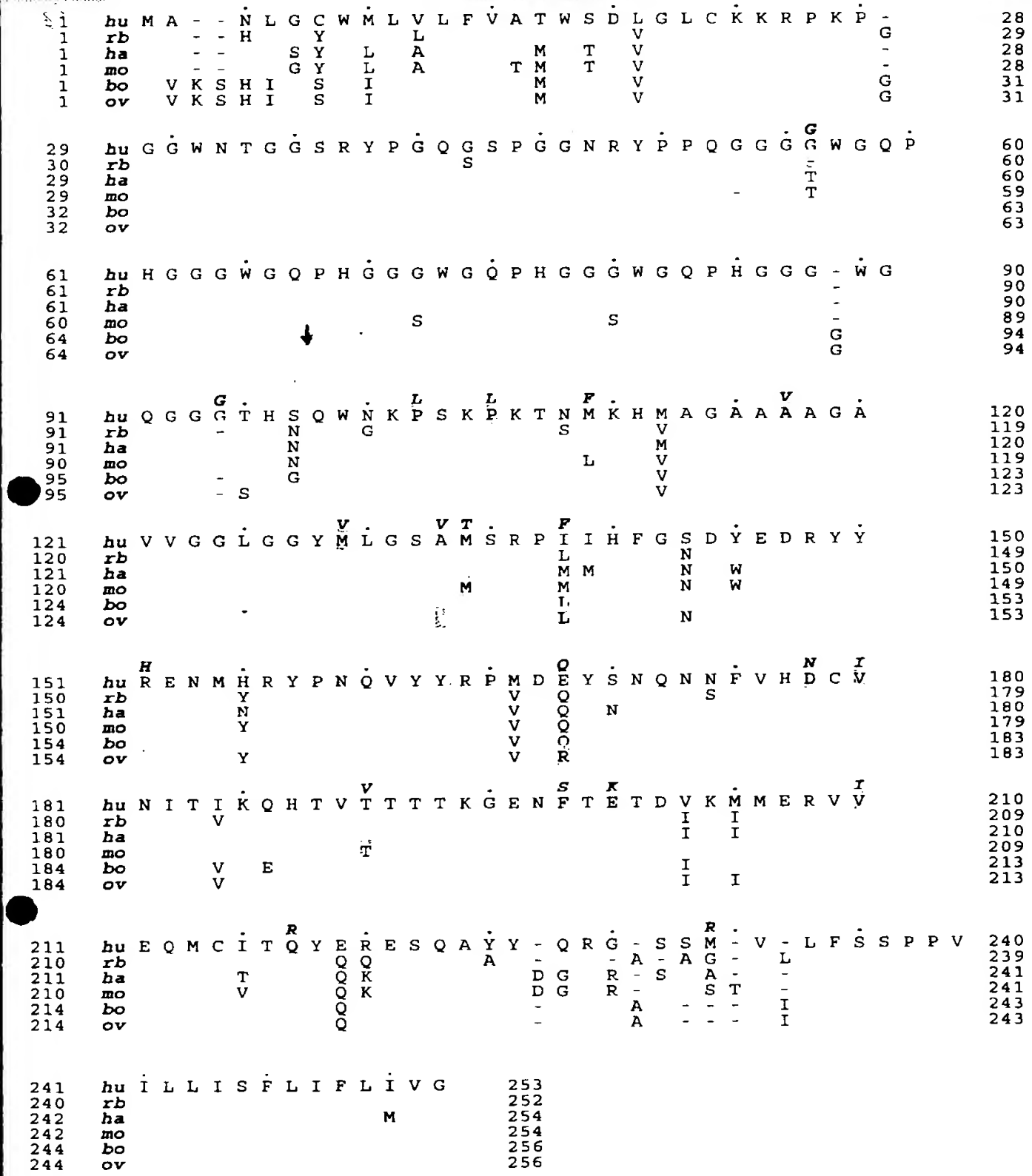
18. A test kit having been provided with means for performing a method according to anyone of claims 8 to 17.

19. A test kit according to claim 18 which is designed for mass-screening purposes

Title: Prion test

Abstract

The invention is related to diagnostic methods for detecting transmissible spongiform encephalopathies (TSE's) such as BSE and scrapie and related disease in humans. The invention provides use of guanidine thiocyanate (gdnSCN) or a functional equivalent thereof for treating at least one sample derived from a mammal including humans for reducing the risk of scoring a false-positive test result in testing said sample for the presence or absence of aberrant prion protein.



Amino acid sequences of human, rabbit, hamster, mouse, cattle and sheep PrP genes. The entire amino acid sequence of human PrP is given; open spaces in the other sequences indicate identity. Polymorphisms are indicated in bold at the top of each block and relate to the shaded positions. ↓ : PHGGGWGQ.

Figure 1

Rabbit antipeptide antisera to parts of the ovine PrP-structure

R521, R522 (to ovine sequence 94-105)

90	*hu*	G	Q	G	G	G	T	H	S	Q	W	N	K P
90	*rb*	G				-			N			G	
90	*ha*	G							N				
89	*mo*	G							N				
94	*bo*	G				-			G				
94	*ov*	G				-	S						

R504, R505, R593-596 (100-111)

97	*hu*	S	Q	W	N	K	P	S	K	P	K	T	N
96	*rb*	N			G								S
97	*ha*	N											
96	*mo*	N											
100	*bo*	G											
100	*ov*												

R568 (126-143)

123	*hu*	G	G	L	G	G	Y	M	L	G	S	A	M	S	R	P	I I H
122	*rb*																L
123	*ha*																M M
122	*mo*												M				M
126	*bo*																L
126	*ov*																L

R532 (145-177)

142	*hu*	G	S	D	Y	E	D	R	Y	Y	R	E	N	M	H	R	Y	P	N	Q	V	Y	Y	R	P	M	D	E	Y	S	N	Q	N	N
141	*rb*		N												Y											V		Q						S
142	*ha*		N		W										N											V		Q		N				
141	*mo*		N		W		-								Y											V		Q						
145	*bo*																									V		Q						
145	*ov*		N												Y											V		R						

R523, R524 (223-234)

220	*hu*	R	E	S	Q	A	Y	Y	-	Q	R	G	-	S
219	*rb*	Q					A		-			-	A	-
220	*ha*	K							D	G		R	-	S
219	*mo*	K							D	G		R	-	
223	*bo*								-				A	
223	*ov*								-				A	

Figure 2

Figure 3

example of test-result with brain-stem extract from sheep

HEEP

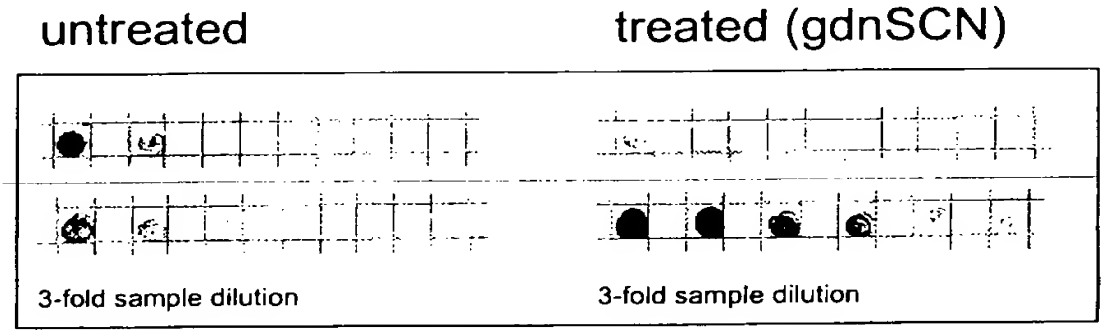


Figure 4

example of test-result with brain-stem tissue from sheep and cattle